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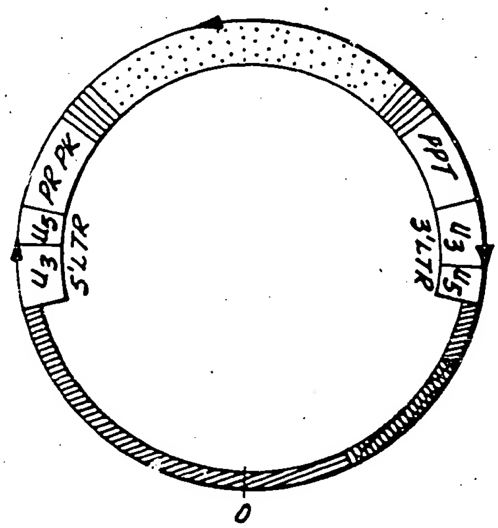
(54) Title: ANTI-SENSE RNA FOR TREATMENT OF RETROVIRAL DISEASE STATES



(57) Abstract

A method for conferring resistance to infection by pathogenic retroviruses. Cells susceptible to infection by a pathogenic retrovirus are transformed with a polynucleotide directing transcription of RNA that is complementary to a region of the retroviral genome and that is efficace to interrupt retroviral replication by substantially preventing formation of pathogenic virions in the treated cell and its progeny. The cells are preferably explanted during the therapeutic transformation step, then introduced into the treated host.

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ANTI-SENSE RNA FOR TREATMENT OF RETROVIRAL DISEASE STATES

Technical Field

This invention relates to genetic engineering methods of treating retroviral disease states.

Background of the Invention

Retroviruses are widespread in nature, and infection with these agents is associated with neoplastic and other disease states in many vertebrate species. Referring to FIGURE 1, the pertinent features of the retroviral life cycle are depicted. The infectious retroviral agent is called a virion. Envelope glycoprotein on the surface of the virion recognizes receptors that mediate entry (1) of two copies of the retroviral genome, each an RNA molecule of between about 8,000 and 10,000 nucleotides, into a target cell. The two genomic virion RNA molecules are copied (2) by a viral reverse transciptase enzyme into duplex linear and circular, supercoiled viral DNA (v-DNA) molecules. The virion RNA first serves as a template for the transcription of a complementary DNA nucleotide sequence (minus strand), and a second-strand DNA copy (plus strand) is then made using the reverse transcribed minus-strand DNA as a template.

Although unintegrated v-DNA can be transcribed (dashed arrow), some of the circular DNA molecules integrate (3) into the cellular genome at a precise point on the viral DNA molecule and a random, or near random, site on host chromosomal DNA. The integrated viral DNA copy is called a provirus.

The pertinent structural elements of a typical provirus are depicted in FIGURE 2. Long terminal repeats (LTRs) containing sequences copied from both ends of viral genomic RNA are located at each end of the DNA provirus and linked directly to host DNA. These LTRs contain regulatory sequences for the expression of the genes required for viral replication: gag, internal structural protein; pol, reverse transcriptase; and env, viral envelope glycoprotein. The LTR regulatory sequences include promoters for the initiation and signals for the termination of transcription. The LTRs usually also include powerful enhancer sequences which amplify the rate of transcription of the viral

genes to the point that proviral RNA transcripts may comprise as much as 0.1 to 1% of total cellular messenger RNA. The transcriptional promoter/enhancer apparatus associated with some retroviruses appears to function only when introduced into particular cell types, resulting in a tissue specific expression of the viral genes.

Referring again to FIGURE 1, the integrated provirus is transcribed (4) into both messenger RNA and full-length genomic virion RNA. The viral messenger RNA is expressed into viral proteins on cellular polysomes (5). The virion RNA contains specific sequences serving as packaging signals for virion assembly (6). Virion RNA and viral proteins are assembled into new virions which bud from the infected host cell.

It is noteworthy that in completing their replicative process, retroviruses usually do not lyse their host cell; and so their life cycle constitutes an efficient mechanism for the introduction and high level expression of genes in living host cells. However, the replication and/or viral gene expression of some retroviruses can have cytotoxic and even cytopathic effects on some types of infected target cells. These pathogenic effects of retroviral infection may lead to systemic disorders associated with disease states.

Human retroviruses have recently been isolated which appear to replicate preferentially in human lymphocytes and so are called human T-cell lymphotrophic viruses (HTLV). For a review, see Wong-Staal and Gallo, Nature 317:395-403, 1985, hereby incorporated by reference. Infection with HTLV type I is associated with the development of a specific type of adult T-cell leukemia whose incidence is presently concentrated in southern Japan and the Carribean basin. Infection with a related retrovirus called HTLV-III (also designated LAV and ARV) is associated with the acquired immunodeficiency syndrome (AIDS). AIDS is emerging as the first major lethal pandemic of the second half of the twentieth century. A third member of this retroviral family, HTLV-II, also infects human T-cells but has not yet been linked to any human disease.

Infection with other standard retroviruses (i.e., encoding the gag, pol, and env genes; but not oncogenes) can induce neoplastic disease in a variety of animal species. For a review, see Pathogenesis of retrovirus-induced diseases, in Molecular Biology of Tumor Viruses: RNA tumor viruses, 2nd Ed., R. Weis, N. Teich, H. Varmus and J. Coffin (eds), New York, Cold Spring Harbor Laboratory, 1984, pp. 785-998, hereby incorporated by reference. Lymphoid leukosis viruses (LLV), including the aetiological agent of avian leukosis, severely impact the poultry industry. Bovine leukemia virus (BLV), which is related to HTVL-I, infects dairy herds causing the disease known as enzootic bovine

leukosis or lymphosarcoma in cattle. The retroviral agent (FeLV) of feline leukemia is also of veterinary concern.

Izant and Weintraub have described the potential of anti-sense (nonsense) DNA strand transcription to inhibit gene activity and suggested that anti-message production can provide a specific molecular "immunity" to the expression of subsequently transcribed genes. Cell 36:1007-1015, 1984.

Summary of the Invention

A method is provided for conferring resistance to infection by pathogenic retroviruses such as those associated with adult T-cell leukemia and acquired immunodeficiency syndrome (AIDS) in humans and with avian leukosis, bovine leukosis and feline leukemia in domestic animals. Pursuant to the invention, cells susceptible to infection by a pathogenic retrovirus are transformed with a polynucleotide directing transcription of RNA that is complementary to a region of the retroviral genome and that is effective to interrupt retroviral replication by substantially preventing formation of pathogenic virions in the treated cell and its progeny. The therapeutic transformation may be achieved with a DNA vector or an RNA vector. The transforming polynucleotide may be selected from the genome of retroviruses known to be the aetiological agents of disease states, including but not limited to HTLV-I, HTLV-II, HTLV-III (LAV, ARV), LLV, BLV, and FeLV. The therapeutically transformed cells may include those susceptible to infection, e.g., T-cells in the case of T-lymphotrophic retroviruses, and also progenitors of such cells, e.g., lymphocytes and hematopoietic stem cells. The therapeutically transformed cells and their progeny are also considered within the scope of the invention. In a preferred embodiment, the cells are explanted during the therapeutic transformation step, then introduced into the treated host.

Brief Description of the Drawings

FIGURE 1 depicts the pertinent features of retroviral replication;

FIGURE 2 depicts the pertinent structural elements of a typical provirus;

FIGURE 3 depicts a representative anti-sense retroviral vector of the invention;

FIGURE 4 shows a representative Northern blot-hybridization of cellular RNA from A-1 cells, as described in Example 1;

FIGURE 5 shows representative dot-blot hybridizations of viral RNA from different sets of cultured chick fibroblasts, as described in Example 2; and

FIGURE 6 shows representative dot-blot hybridizations of viral RNAs as described in Example 4.

Detailed Description of the Preferred Embodiment

We describe here a model system employing retroviral vectors which demonstrates effects of anti-sense RNA on steps in retroviral replication independent of viral gene expression. The model system is predicated on the discovery that anti-sense RNA for specific cellular genes, when introduced via retroviral vectors to transform a target cell, may not suppress the expression of the cognate cellular gene when there are high steady state levels of RNA from the cellular gene present in the target cell cytoplasm. This unexpected observation results, in large measure, because RNA transcripts from the cellular gene, being anti-sense to the incoming retroviral RNA, markedly inhibit infection by the retroviral vector. These findings provide solid experimental support for a novel application of anti-sense RNA in anti-viral therapy, and establish a rational basis for a therapeutic approach to retrovirus induced disease in humans such as acquired immune deficiency syndrome (AIDS) caused by infection with HTLV-III and/or related retroviruses, e.g., LAV and ARV.

Pursuant to the invention, a cell susceptible to infection by a pathogenic retrovirus is transformed with a polynucleotide directing transcription of RNA that is complementary to part of the genomic virion RNA of the targeted pathogenic retrovirus and that effectively interrupts retroviral replication in the transformed cell and its progeny. By "polynucleotide" is meant a relatively small natural or synthetic nucleic acid polymer containing from many thousand to a minimum of about 10 to 20 nucleotide bases. By "transformation" is meant the process of changing the genotype of a recipient cell mediated by the introduction of DNA, RNA, or nucleotide analogue polymer.

The transforming polynucleotide can be a double-stranded DNA molecule whose sense strand is homologous to a region of the targeted virion RNA. Alternatively, the cell can be treated by transformation with an RNA vector containing a polynucleotide that is complementary to a region of the targeted virion RNA. For example, the RNA vector can be a therapeutic retroviral construct, in which case the v-DNA reverse transcribed from the latter polynucleotide, once integrated into the cellular genome, will transcribe RNA that is complementary to the targeted virion RNA. Whether the transforming polynucleotide is introduced into the cell via a DNA vector or an RNA vector, the RNA transcript directed by the transforming polynucleotide must contain base sequences complementary to the virion RNA of the pathogenic

retrovirus, that is, the (anti-sense) RNA directed by the transforming polynucleotide and the targeted virion RNA must be capable of forming molecular hybrids.

The RNA directed by the transforming polynucleotide must in addition interrupt replication of the targeted pathogenic retrovirus in the transformed cell, that is, the transforming polynucleotide must confer resistance to infection by preventing or reducing the formation of pathogenic virions in the transformed cell. It is considered therapeutically efficacious to select a transforming polynucleotide that will interrupt replication by substantially preventing formation of pathogenic virions in the treated cell and its progeny. The anti-sense RNA directed by the transforming polynucleotide is preferably selected to interrupt retroviral replication by disrupting the formation and/or processing of viral DNA and thereby prevent its integration into the cellular genome. The selection of a transforming polynucleotide effective to therapeutically interrupt retroviral replication in accordance with this disclosure is achievable by standard genetic engineering manipulations such as those described below. By including a suitable promoter and other essential regulatory sequences with the polynucleotide in a transforming vector, the therapeutically transformed cell synthesizes the anti-sense RNA transcripts at a continuous rate, and so a steady-state level of resistance to infection is conferred on the cell and its progeny.

The observations described in the Examples provide a clear rationale for an anti-sense RNA based strategy for the amelioration of the pathogenic effects of human retroviruses such as HTLV-III/LAV/ARV induced immune deficiency or AIDS. These human T-lymphotropic retroviruses must infect host T-cells to exert their pathogenic effects, and their life cycle within those target cells is in no relevant way different from that of the model viruses described below, that is, the known differences would not be expected to render human retroviral replication insensitive to the effects observed in the model system. An anti-sense RNA therapy for AIDS and pre-AIDS syndromes is contemplated as follows:

From a DNA clone of the HTLV-III/LAV genome a series of discrete segments are inserted, in an anti-sense orientation, into a prepared cloning site of an engineered vector, for example, one recently developed from murine ecotropic and amphotropic retroviruses for use in a wide range of animal cells including human cells. A suitable vector is described by Miller, Law and Verne in Mol. Cell Biol. 5:431-432, 1985, hereby incorporated by reference. A representative anti-sense retroviral vector is shown in FIGURE 3. The vector

may be constructed in the form of a bacterial plasmid for efficient production in large quantities. Here the bacterial region (indicated by oblique hatching) includes a replication region (o) and an antibiotic drug resistant gene (cross-hatched region) for effective selection. The remaining retroviral region includes the 5' LTR (u3 and u5) that provides the enhancer and promoter sequences for RNA transcription, the tRNA primer attachment site (PR), the packaging sequences (PK), the polypurine tract (PPT) important for reverse transcription, and the 3' LTR (u3 and u5) that provides the termination signals of RNA transcription. An engineered polylinker region (transversely hatched regions) provides convenient restriction enzyme sites for inserting any specific anti-sense polynucleotide (dotted region). (The opposite orientations of the retroviral genes and the anti-sense insert are indicated by arrowheads). The anti-sense polynucleotide should not be homologous to the retroviral replicative genes in a helper virus or packaging cell line used to rescue the resistance-conferring segment(s), below.

The engineered vector is introduced by standard gene transfer technology into cell lines that are potentially susceptible to infection by the pathogenic retrovirus, and transformants are selected, e.g., with a drug resistance marker within the retroviral construct or contransferred with the construct. Transformants are tested and selected for high steady state levels of RNA molecules anti-sense with respect to the targeted retroviral genomic RNA.

Cell lines expressing high steady-state levels of RNA anti-sense to various segments of the pathogenic retroviral genome are tested for their resistance to infection by the AIDS inducing retroviruses, e.g., as described for the model viruses below. Cell lines containing retroviral constructs most effective in inhibiting retroviral replication are identified.

The identified anti-sense RNA containing constructs are then rescued as replication defective viruses using available packaging systems such as that referenced above which provide high titer virus stocks of both the helper virus free and helper virus containing varieties.

Clinical trials using the selected anti-sense polynucleotides can follow two general strategies. A first strategy involves inoculating patients having AIDS or pre-AIDS with vector stocks containing helper virus. The vector would infect patient T-cells not yet harboring the AIDS virus, and expression of the anti-sense polynucleotide would render the therapeutically transformed cells resistant to infection by the pathogenic retrovirus. This strategy would stop or retard the progression of the disease, and the resistant T-cells or other stem cells might serve to reconstitute the patient's immune system. Problems could

arise, however, from a lack of T-cells and/or stem cells which do not harbor the pathologic retrovirus and from an inability of the anti-sense vector to reverse the expression of the AIDS virus in previously infected cells (as predicted by the test results described below). There is an additional risk of unknown pathogenic effects resulting from the replication of the vector and/or helper retrovirus in the human host.

A second strategy avoids the potential disadvantages associated with the above in vitro innoculation. Precursors to T-cells (e.g., marrow stem cells, fetal thymus) are explanted from the patient (or from allogeneic or isogeneic donors), infected at high multiplicity by helper virus free stocks of the anti-sense vector, and transplanted into the patient using available transplantation technology. Cells successfully infected with the anti-sense vector could reconstitute the patient's immune system with T-cells resistant to infection by residual pathogenic AIDS virus. The use of a cytotoxic drug resistance gene within the vector may enhance the immune reconstitution by permitting in-vivo selection of cells carrying the anti-sense vector. This transplantation therapy avoids the risk of vector or helper virus induced pathogenicity because the anti-sense vector cannot spread outside of the initially infected cells and their progeny.

The following Examples are provided to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. The Examples also indicate how the seminal observation that the anti-viral activity of anti-sense RNA molecules resident in host cells does not work primarily through the anti-sense inhibition of (viral) gene expression resulted from a failed experiment. The Examples are not intended in any way to otherwise limit the scope of the disclosure and the protection granted by Letters Patent hereon.

EXAMPLE 1

Cell test system.

To test the effects of anti-sense RNA molecules on the expression of genes such as oncogenes that are typically expressed at very high levels, the gene neo^R, which confers on eukaryotic cells resistance to the cytotoxic effects of the neomycin analog G418 (Southern and Berg, J.Mol.App.Genetics 1:327-341, 1982), was introduced on an avian retroviral vector by transfection into a Japanese quail cell line. (The gene neo^R was chosen as a stalking horse in this model system because, unlike natural oncogenes, neo^R has no natural counterparts in higher animal cells; thus, any inhibition of neo^R expression elicited by the anti-sense manipulations described below would be free of background noise

incidental to any cellular transcripts). Colonies of transformed QT-35 cells resistant to the lethal effects of G418 were expanded in culture and tested by "northern" blot-hybridization analysis for the presence of high levels of RNA molecules transcribed from the $\underline{neo}^R$ gene. One cell line designated A1 was selected as the test system for further study. FIGURE 4 shows a representative Northern blot-hybridization analysis of cellular RNA from the A-1 cells. Cellular RNA from G418 resistant A-1 cells (lane b) and from control quail QT35 cells (lane a) were extracted, then denatured and subjected to formaldehyde-agarose gel electrophoresis. The RNAs separated on the gel were blotted onto nitrocellulose filters and hybridized to $\underline{neo}^R$ probes.

EXAMPLE 2

Construction of retroviral vectors expressing neo^R RNA in sense and anti-sense orientations.

Retroviral vectors containing the $\underline{neo}^R$ gene in either the sense or anti-sense orientation were prepared using the 779NC TAQ26 system described by Hughes and Kosik in Virology 136:89-99, 1984. The 779NC TAQ26 system consists of an infectious DNA clone of the Schmidt-Rupin strain of Rous sarcoma virus subgroup A (SR-TSV-A) from which the src gene has been deleted. We inserted the $\underline{neo}^R$ gene in either the sense or anti-sense orientation at an engineered Cla-1 restriction endonuclease cleavage site so that the inserts would be transcribed from the vector as spliced messenger RNAs in the same fashion as the src gene in the wild type SR-RSV-A. Sense and anti-sense $\underline{neo}^R$ vectors were then recovered as virions from supernatant fluids of chick embryo fibroblasts (CEF) transfected with the engineered constructs. The replication of the recovered viruses on fresh cultured cells was assayed by detection of $\underline{neo}^R$ sense or anti-sense sequences in virion RNA released from infected cells using strand specific hybridization probes.

Both sense and anti-sense inserts remained equally stable within the retroviral vectors through many rounds of replication. The vector containing $\underline{neo}^R$ in the sense orientation conferred resistance to G418 on infected cells in culture while, as expected, the analogous vector with $\underline{neo}^R$ in the anti-sense orientation did not confer such resistance. The sense $\underline{neo}^R$ vector was designated N-10, and the anti-sense $\underline{neo}^R$ vector was designated α N-10.

FIGURE 5 shows representative dot-blot hybridizations of viral RNA from different sets (rows a-f) of cultured chick fibroblasts. Supernatant fluid from each culture was collected and virions purified through sucrose gradients and pelleted by centrifugation. Viral RNA was extracted, serially diluted and then blotted on nitrocellulose filters and hybridized to strand specific

probes designed to detect either the anti-sense neo^R gene (plate A) or the sense neo^R gene (plate B) in the viral genomes. Rows a-c are viruses from chronically infected chick fibroblasts, specifically: a, N-10; b, N-10 + G418 treatment; c, α N-10. Rows d-f are the original stocks of virus from transfected fibroblasts, specifically: d, α N-10; e, N-10; f, virus derived from the vector (only) as a control.

EXAMPLE 3

Effect of the neo^R anti-sense vector on G418 resistance in A1 cells.

Since A1 cells depend upon neo^R expression for viability in the presence of G418, we tested the effects of infection of A1 cells with α N-10 (which should express high levels of anti-sense neo^R transcripts) on cell viability in presence of G418. Surprisingly, we were unable to detect any effect of α N-10 infection on the viability of A1 cells in the presence of G418, even using very sensitive assays such as cloning efficiency in soft agar. Thus, this anti-sense vector apparently failed to suppress the expression of the target gene.

EXAMPLE 4

Effect of the neo^R transcripts in A1 cells on the replication of the neo^R anti-sense vector in A1 cells.

The reason for the failure of α N-10 to suppress neo^R expression in A1 cells became apparent when we assayed the supernatant fluids from infected A1 cells for the presence of virus. By the use of the same probes described in conjunction with Example 2 we determined that the level of α N-10 virus containing anti-sense neo^R sequences was more than 100-fold lower than the level of virus produced from A1 cells infected with control N-10 virus containing neo^R in the sense orientation. However, when probed for viral sequences other than anti-sense neo^R (e.g., viral long terminal repeat or LTR sequences) we detected significant production of virus from α N-10 infected A1 cells.

These results are shown in FIGURE 6. Dot-blot hybridizations of viral RNAs were performed as described in Example 2 but using LTR probes to detect total viral genomes (panels A) as well as single-strand probes to detect anti-sense neo^R genes (panel B) and single-strand probes to detect sense neo^R genes (panel C). Rows a-c represent viruses harvested from G418 treated quail A-1 cells infected with: a, N-10; b, α N-10; and c, vector control. Rows a-f are original viral stocks from chicken fibroblasts transfected with: d, N-10; e, vector control; and f, α N-10.

These serendipitous findings indicate that α N-10 virus loses its anti-sense neo^R gene when infecting A1 cells and/or that anti-sense neo^R containing viruses are strongly inhibited during replication on A1 cells. Since an

analogous loss did not occur during infection of A1 cells with control N-10 virus we conclude that the observed loss results from the high levels of neo^R RNA molecules in A1 cells which are anti-sense with respect to α N-10 sequences (but, conversely, identical to N-10 sequences). In agreement with this conclusion, virus derived from the vector itself, without insertions of any neo^R related sequences, replicated well on A1 cells.

The above test results indicate that anti-viral activity of anti-sense RNA molecules resident in host cells does not work primarily through the anti-sense inhibition of (viral) gene expression, i.e., pathway (5) in Fig. 1, as demonstrated by the fact that the neo^R sequence in α N-10 does not constitute a gene whose expression is required for viral replication. Therefore, anti-sense RNA molecules must block other steps in viral replication. Excision of anti-sense neo^R sequences from α N-10 by neo^R sense RNA molecules would suggest that the affected replication step is early in the viral life cycle, most probably during the formation and/or processing of viral DNA molecules before their integration into chromosomal DNA. Hybrids between anti-sense RNA molecules and complementary virion RNA sequences or plus strand v-DNA sequences provide a plausible substrate for the excision of specific sequences we have observed. Other steps in retroviral replication could also be inhibited by the anti-sense RNA, but presumably not viral gene expression.

In α N-10 infected A1 cells, viral nucleic acid molecules which had lost their anti-sense neo^R sequences were still able to replicate as viruses because the deletion mediated by the anti-sense RNA did not affect any of the viral genes (gag, pol, env) that are required for replication. If the anti-sense RNA molecules in the cell include complements to any or all of these viral replication genes, defective viruses should result which could not replicate beyond the first round of infection (unless provided with a competent helper and/or exposed to overlapping defectives which could lead to repair by recombination).

While the present invention has been described in conjunction with a preferred embodiment and specific examples, the description is intended to illustrate the invention and is not meant to limit it, unless such limitation is necessary to avoid the pertinent prior art. One of ordinary skill after reading the foregoing specification will be able to effect various changes, substitutions of equivalents, and other alterations to the methods and compositions set forth herein. Therefore, the protection granted by Letters Patent should be limited only to the definition contained in the appended claims and equivalents thereof.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of conferring resistance to retroviral infection comprising the step of transforming a cell susceptible to infection by a pathogenic retrovirus with a polynucleotide directing transcription of RNA complementary to the virion RNA of the pathogenic retrovirus and effective to interrupt retroviral replication.

2. The method of Claim 1 wherein the transformation is by DNA vector means.

3. The method of Claim 1 wherein the transformation is by RNA vector means.

4. The method of Claim 3 wherein the transformation is by a retroviral construct.

5. The method of Claim 1 wherein the pathogenic retrovirus is selected from among the aetiological agents of acquired immunedeficiency syndrome (AIDS), adult T-cell leukemia, avian leukosis, bovine leukosis, and feline leukemia.

6. The method of Claim 5 wherein the pathogenic retrovirus is the aetiological agent of AIDS.

7. The method of Claim 1 wherein the pathogenic retrovirus is selected from among HTLV-I, HTLV-II, HTLV-III, LAV, ARV, LLV, BLV, and FeLV.

8. The method of Claim 1 wherein the pathogenic retrovirus is a T-lymphotrophic virus.

9. The method of Claim 8 wherein the pathogenic retrovirus is selected from the AIDS-associated retrovirus family.

10. The method of Claim 1 wherein the cell is selected from among T-cells, thymus cells, lymphocytes, and hematopoietic stem cells.

11. The method of Claim 1 wherein the cell is a human cell.

12. A cell transformed by the method of Claim 1.

13. Progeny of the cell of Claim 12.

14. A cell transformed by the method of Claim 1 containing the polynucleotide detectable by solid phase hybridization assay.

15. Progeny of the cell of Claim 14.

16. A cell transformed by the method of Claim 1 containing RNA transcripts of the polynucleotide detectable by solid phase hybridization assay.

17. Progeny of the cell of Claim 16.

18. A vector useful for transforming eukaryotic cells to confer resistance to infection by a pathogenic retrovirus, comprising a polynucleotide directing transcription of RNA complementary to the virion RNA of the pathogenic retrovirus in a construct capable of transforming eukaryotic cells susceptible to infection by the pathogenic retrovirus.

19. A method of conferring resistance to infection by a pathogenic retrovirus comprising the step of introducing the vector of Claim 18 into a eukaryotic host.

20. A method of conferring resistance to infection by a pathogenic retrovirus comprising the steps of transforming explanted cells with the vector of Claim 18 and introducing the transformed cells into a eukaryotic host.

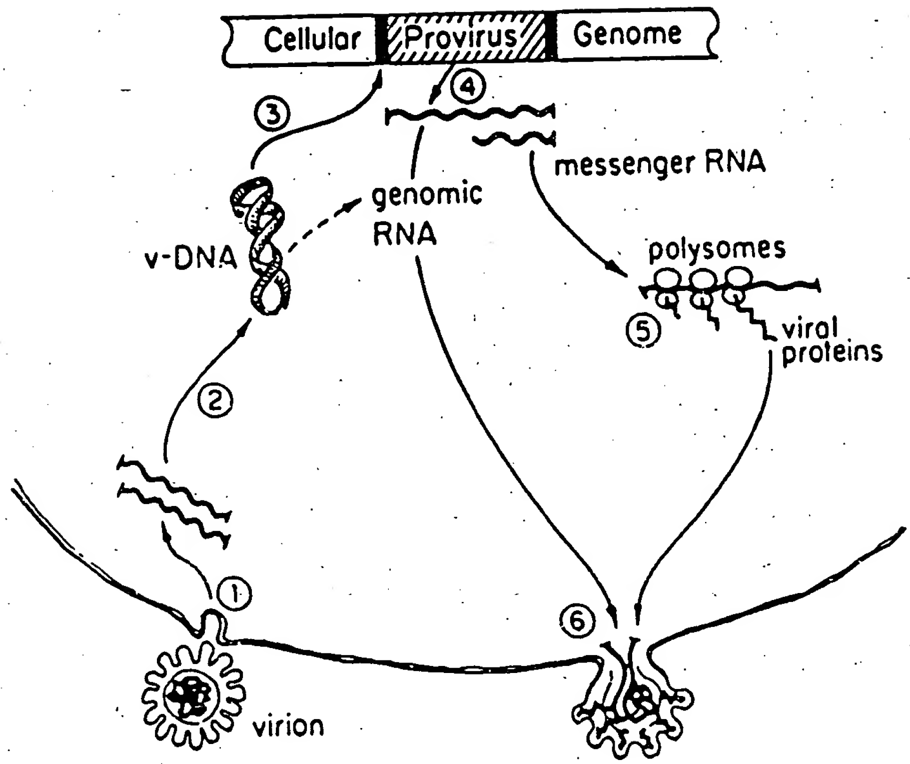

Fig. 1.

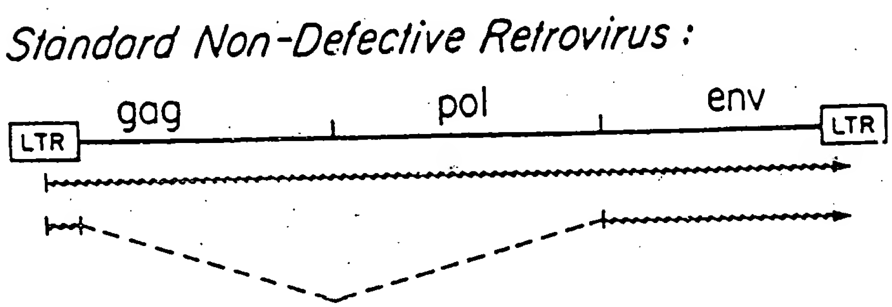

Fig. 2.

SUBSTITUTE SHEET

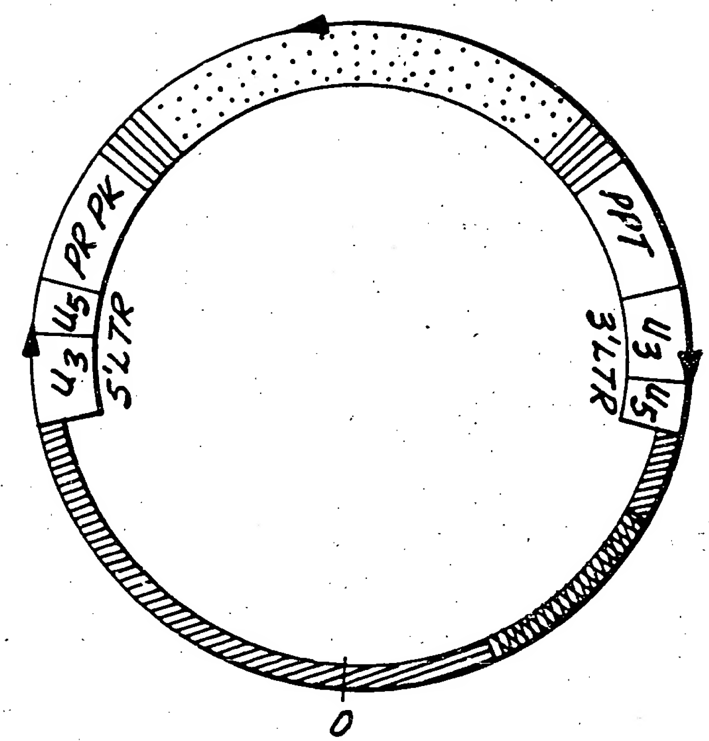

Fig. 3.

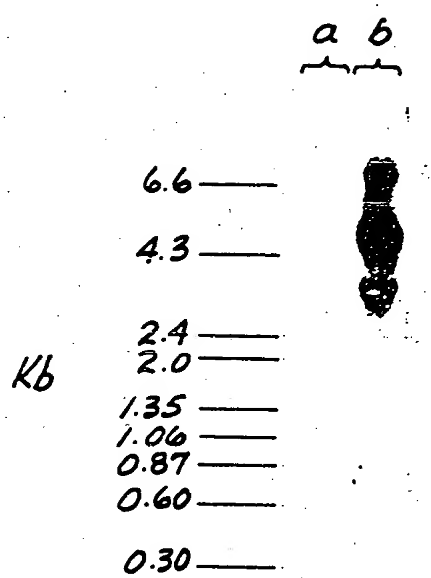

Fig. 4.

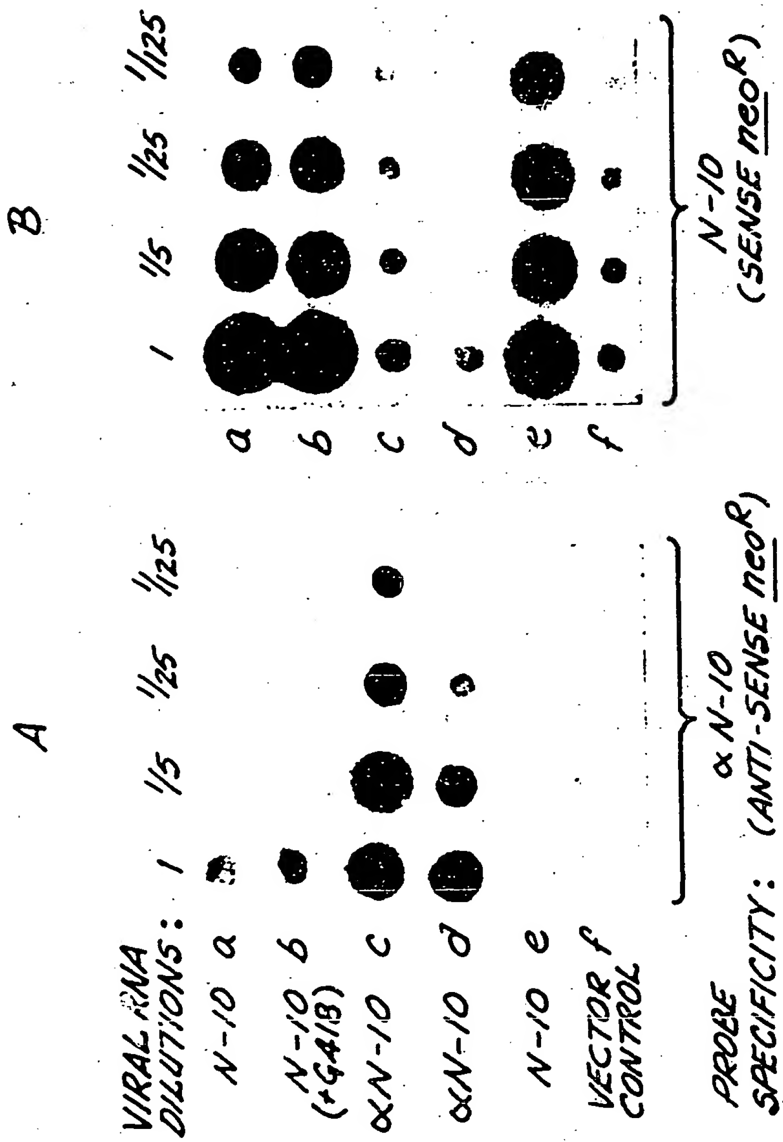
A
B
VIRAL RNA DILUTIONS:
1
1/5
1/25
1/125
N-10 a
N-10 (+G418) b
αN-10 c
αN-10 d
N-10 e
VECTOR CONTROL f
PROBE SPECIFICITY:
αN-10 (ANTI-SENSE neoR)
N-10 (SENSE neoR)
a
b
c
d
e
f

Fig. 5.

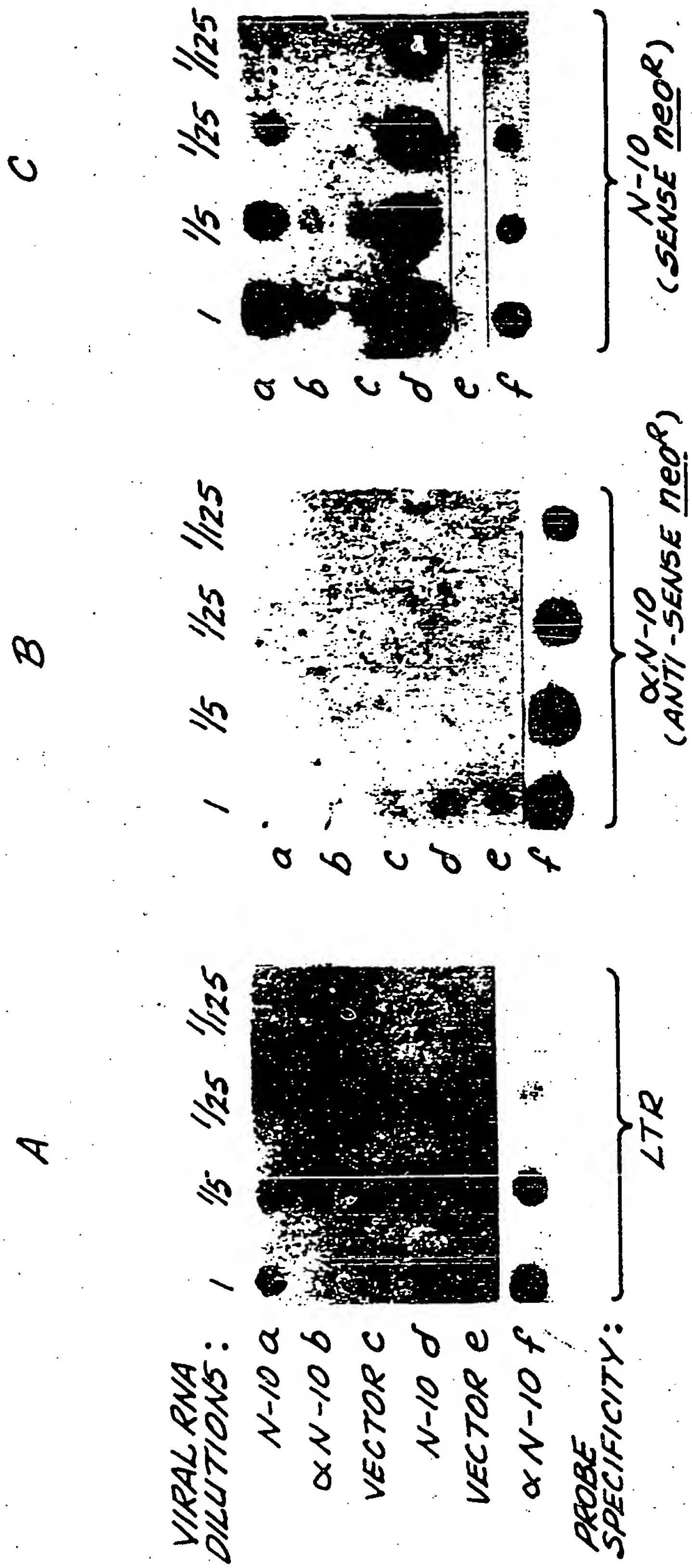
A
B
C
1
1/5
1/25
1/125
VIRAL RNA DILUTIONS:
N-10 a
αN-10 b
VECTOR c
N-10 d
VECTOR e
αN-10 f
PROBE SPECIFICITY:
LTR
αN-10 (ANTI-SENSE neo^R)
N-10 (SENSE neo^R)
a b c d e f

Fig. 6.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/02604

I. CLASSIFICATI N F SUBJECT MATTER (if severa. classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A01N 63/00, A61K 35/76, C12N 15/00, C12N 5/00
U.S. CL: 424/93

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/93 435/172.3,240,241,317,948 935/22,24,32,52-55,57,59,65,66,70,71

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

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III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,P Y,P	Nature, Volume 318, issued 05 December 1985, E.C.M. Mariman, New Strategies for AIDS Therapy and Prophylaxis", see page 414.	1-19 20
X,P Y,P	Nature, Volume 318, issued 05 December 1985, R. Tellier et al., New Strategies for AIDS Therapy and Prophylaxis," see page 414.	1,2,4-20 3
Y	Science, Volume 229, issued 26 July 1985, J.G. Izant et al., "Constitutive and Conditional Suppression of Exogenous and Endogenous Genes by Anti-Sense RNA," see pages 345-352.	1-20

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
18 Feb 1987	02 MAR 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Margaret Moskowitz Margaret Moskowitz

International Application No. PCT/US86/02604

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No[18]
Y	Nature, Volume 313, issued 21 February 1985, U.B. Rosenberg et al., "Production of Phenocopies by Kruppel Antisense RNA Injection into Drosophila Embryos," see pages 703-706.	1-20
Y	Proceedings of the National Academy of Sciences, USA, Volume 82, issued January 1985, D.A. Melton, "Injected Anti-sense RNAs Specifically Block Messenger RNA Translation _In Vivo_," see pages 144-148.	1-20
Y	Virology, Volume 136, issued 15 July 1984, S. Hughes et al., "Mutagenesis of the Pegion between Env and Src of the SR-A Strain of Rous Sarcoma Virus for the Purpose of Constructing Helper-Independent Vectors, "see pages 89-99.	1-20
Y	Cell, Volume 37, issued July 1984, C.L. Cepko et al, "Construction and Applications of a Highly Transmissible Murine Retrovirus Shuttle Vector," see pages 1053-1072.	1-20

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